Thesis for the Degree of Master of Science

Characterization of Ghrelin gene of *Paralichthys olivaceus*, olive flounder

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August 2010

Characterization of Ghrelin gene of *Paralichthys olivaceus*, olive flounder

양식 넙치의 Ghrelin 유전자의 특성

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부경대학교

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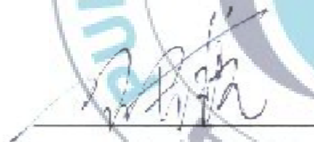

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Characterization of Ghrelin gene of *Paralichthys olivaceus*,
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ABSTRACT

Ghrelin is brain – gut peptide with growth hormone – releasing and appetite – inducing activities. It is mainly secreted from the stomach but it is also expressed widely in different tissues and therefore may have both endocrine and paracrine effects. Ghrelin is the endogenous ligand of the G protein-coupled growth hormone secretagogue receptor.

The ghrelin gene is expressed most abundantly in stomach. The

mRNA is also detected in other tissues and cell lines. However, the mechanism of the transcriptional regulation of the ghrelin gene has not yet been clarified.

We now report the purification and identification in flounder stomach of an endogenous ligand specific for GHR-S. It is a ghrelin. ghrelin is a peptide of 28 amino acids, in which the serine 3 residue is *n*-octanoylated. The acylated peptide specifically releases GH both in vivo and in vitro, and O-n-octanoylation at serine 3 is essential for the activity. Human ghrelin is homologous to rat ghrelin apart from two amino acids. The occurrence of ghrelin in both rat and human indicate that GH release from pituitary may be regulated not only by hypothalamic GHRH, but also by ghrelin.

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INTRODUCTION

In December 1999, an endogenous ligand of the GHS-R type 1a was first reported (Kojima *et al.*, 1999). The ligand was given the name ghrelin from the Proto-Indo-European word of 'ghre', which means grow, and 'relin' as it has GH-releasing activities. Ghrelin is a 28 amino acid peptide with a fatty acid chain modification on the N-terminal third amino acid (Korbonitits *et al.*, 2004). Interestingly, at the same time a stomach-derived mRNA sequence was identified coding for a protein with sequence similarities to motilin and named motilin-related peptide m46 (Tomasetto *et al.*, 2000). This peptide later turned out to be identical to ghrelin, although the fatty acid modification was not recognized (Kojima *et al.*, 2000). Monitored calcium concentration changes in a CHO (Chinese Hamster Ovary) cell line stably transfected with human GHS-R 1a and found activity in stomach extracts, while searched for genes selectively expressed in stomach tissue (Fuglsang *et al.*, 2006).

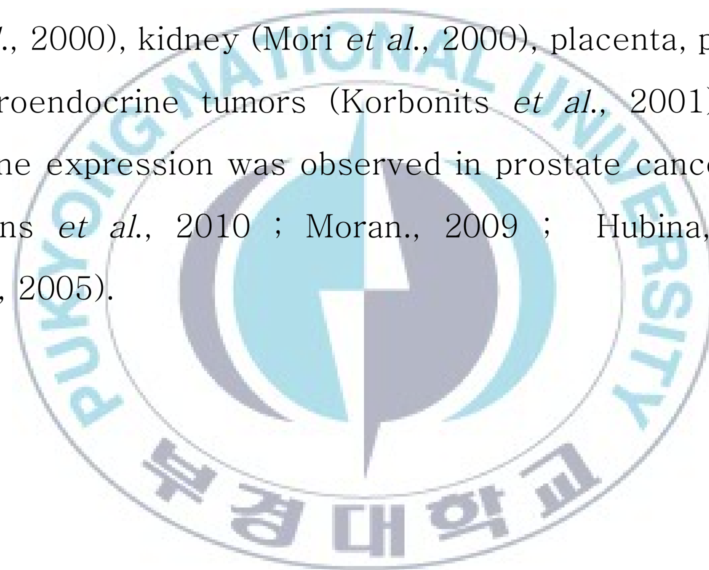
High pressure liquid chromatography (HPLC) and mass spectrometry were used to identify the amino acid sequence of ghrelin and a discrepancy between the observed and calculated molecular weight pointed to the presence of a post-translational

modification. The hydrogen atom of the hydroxyl group of the third N-terminal amino acid serine residue is replaced by a hydrophobic moiety, $C_7H_{15}CO$, in other words the hydroxyl group of Ser^3 is octanoylated. No other naturally occurring peptide has been previously shown to have this acyl group as a post-translational modification. Asplice variant of ghrelin with 27 amino acids missing the 14th amino acid (glutamine) was also identified. Later, biologically active analogue of ghrelin were described in much smaller amounts with acyl chains of 10 or 11 Carbons or with a peptide chain with the amino acid at position arg^{28} (arginine, the last amino acid in mature ghrelin) missing (Honda *et al.*, 2000). The n-octanoyl group at the Ser3 of the ghrelin molecule seems to be essential for some of the hormone's bioactivity, including GH release and appetite. Non-acylated (desoctanoyl or desacyle) ghrelin circulates in far greater amounts than the acylated form and does not displace ghrelin from its hypothalamic and pituitary binding sites (Honda *et al.*, 2000), and is unable to stimulate GH release in vivo in rats and humans (Bowers., 1977). However, increased numbers of studies report biological effects of desoctanoyl ghrelin (Moran *et al.*, 2009 ; Bedendi *et al.*, 2003 ; Cassoni *et al.*, 2001 ; Nanzer *et al.*, 2004 ; Tompson *et al.*, 2004).

Ghrelin has been isolated from the stomach and identified as an endogenous ligand for the growth hormone (GH) secretagogue receptor (Kojima and Kangawa., 2008). In rodent, ghrelin has been

shown not only to stimulate GH secretion (Date *et al.*, 2000) but also to exert orexigenic and adipogenic effects (Asakawa *et al.*, 2001 ; Tschop *et al.*, 2000). In addition to its endocrine activities, ghrelin protects heart function from experimentally induce cardiac heart failure *in vivo* (King *et al.*, 2001). Effects of GH secretion and feeding behavior are also observed in humans (Berthold *et al.*, 2010).

Ghrelin gene has been shown to be abundantly expressed in the stomach, lower levels of the expression have also been demonstrated in the hypothalamus (Kojima *et al.*, 1999), intestine (Date *et al.*, 2000), kidney (Mori *et al.*, 2000), placenta, pituitary and other neuroendocrine tumors (Korbonits *et al.*, 2001). Recently, ghrelin gene expression was observed in prostate cancer cell lines (Schellekens *et al.*, 2010 ; Moran., 2009 ; Hubina, Goth, and Korbonits., 2005).



MATERIALS AND METHODS

MATERIALS

Fish tissues

The stomach tissues were obtained from mature olive flouner (*Paralichthys olivaceus*; n=8; size; 40 ± 10cm , body weight; 1000 ± 200 g ; 3years old) and stored at -70℃ until used.

Bacteria strain

The *E. coli* strain XL1-Blue [F'::Tn10 *pro*A^+B^+ *lac*I^q Δ(lacZ) M15/*recA1 endA1 gyr*A96(Nal^r) *thi hsd*R17(r_k^- m_k^+) *supE44 relA1 lac*] was used for transformation and color selection.

Enzymes

Restriction enzymes were purchased from Promega (USA), Amersham Bioscience (UK) and TAKARA (Japan). Taq polymerase and pfu polymerase were obtained from Bioneer (Korea) and Clontech (USA). Reverse transcriptase were obtained from Invitrogen (USA).

Oligonucleotide primers

The primer for ghrelin gene cloning were designed on the basic of the conserved sequences from Known ghrelin gene of different species (Zebrafish, European seabass, Mozambique tilapia and others) and synthesized from Bioneer (Korea)

Other materials

The primer walking system (DNA walking SpeedUp™ premix Kit) was purchased from Seegene (Korea). The RACE system (BD SMART™ RACE cDNA Amplification Kit) was purchased from Clontech (USA). The plasmid isolation, gel and PCR purification system were purchased from Bioneer (Korea) and Promega (USA). The mRNA iolation Kit (Micro-FastTrack 2.0™) was purchased from Invitrogen (USA). The TRIzol reagent for total RAN isolations was purchased from Difco (USA).

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Table 1. Oligonucleotide primers used for the study

Name	Sequence	Remark
ghr-CODEHOP-F	5’ - CCTCCTTCCTGTCCCCCwsncaraarcc - 3'	Ghrelin degenerated, Forward
ghr-CODEHOP-R	5’ - TCATGGTCATGCCCATCtcRaanggngc - 3’	Ghrelin degenerated, Reverse
Ghr-3’ RACE-GSP1	5’ - GAACAAGGGGAAACCTCCCAGAC - 3’	Ghrelin 3’ RACE GSP1
Ghr-3’ RACE-GSP2	5’ - CGAGGACCACCACATCACAGTAAGT - 3’	Ghrelin 3’ RACE GSP2
Ghr-5’ RACE-GSP1	5’ - ACTGATGCTTTATTTTGACCACC - 3’	Ghrelin 5’ RACE GSP1
Ghr-5’ RACE-GSP2	5’ - TTGAGATGTGGTTGAAAGCTCTG - 3’	Ghrelin 5’ RACE GSP2
Ghr-5’ RACE-GSP3	5’ - TAAAGCAGTAAATCTGCCAGTCG - 3’	Ghrelin 5’ RACE GSP3
Ghr-specific-F	5 ‘- TTAACGCTATACGTTGCTTCGTC - 3'	Ghrelin specific primer, forward.
Ghr-ext-R1	5’ - GTCGACTTACACCACAAGGTCAG - 3’	Ghrelin primer extension R1
Ghr-ext-R2	5’ - GCCTFFTFTTTCTTTTCAGAAAC - 3’	Ghrelin primer extension R2

METHOD

Isolation of RNA

Total RNA isolation was extracted from the olive flounder stomach using TRIzol reagent (Difco, U.S.A).

100 mg of the fresh brain tissue was placed in liquid nitrogen and grinded to a fine powder with mortar and pestle under liquid nitrogen, placed them within 1mℓ of TRIzol reagent per 50 mg of tissue. The lysate was incubated on ice for 5 min and 200 μℓ of chloroform was added, mixed vigorously by vortex mixer for 15 sec and incubated once for 5 min. the sample was then centrifuged at 14,000 rpm for 15 min at 4 ℃. Equal volume of chloroform was added and mixed vigorously by vortex mixer for 15 sec and incubated on ice for 15 min. the sample was centrifuged at 14,000 rpm for 15 min at 4 ℃ and aqueous phase was transferred to a new tube. 500 μℓ of isopropanol was added, mixed by vortexing and incubated for 15 min on ice. Chilled the sample tube on ice was centrifuged at 14,000 rpm for 5 min at 4 ℃ and the supernatant was discarded. The RNA pellet was washed with 1 mℓ of 75 % ice-cold ethanol and centrifuged at 14,000 rpm for 5 min at 4 ℃. The pellet was dried briefly for 10 min on ice and dissolved in 20 μℓ of DEPC-treated water. The quality of RNA was estimated RNA was

use for cDNA synthesis.

Poly(A) RNA was isolated using a Micro-FastTrack 2.0™ (Invitrogen). 10 ㎕ (500 ㎍) of purified poly(A) RNA was aliquoted to a new microcentrifuge tube and 1㎖ of Micro-FastTrack 2.0™ lysis buffer was added. The mixed sample tube was heated at 65 ℃ for 5 min and placed immediately for 1 min on ice. 63 ㎕ of 5 N NaCl was added, and mixed well with the oligo-T cellulose for 2 min. the poly(A) RNA was isolated with a method described in Micro-FastTrack 2.0™ (Invitrogen) manual. The quality of poly(A) RNA was estimated by electrophoresis on 2 % agarose gel. The isolated poly(A) RNA was used to BD SMART™ RACE cDNA amplification kit (Clontech) for ghrelin gene.

cDNA synthesis

Reverse transcriptase PCR was used to SuperScript™ II Reverse Transcriptase system (invitrogen). Add the 1 ㎕ of oligo$(dT)_{12-18}$(500 ㎍/ ㎖), 1 ng to 5 ㎍ of total RNA, 1 ㎕ dNTP mix (10 mM each) and fill the nuclease free water to total 12 ㎕ reaction volume. Heat mixture to 65 ℃ for 5 min and quick chill on ice. Collect the contents of the tube by brief centrifugation and add the 4 ㎕ of 5 X First-strand buffer, 2 ㎕ of 0.1 M DTT and 1 ㎕ of RNaseOUT™ (40 units/ ㎕). Then mix contents of the tube gently, and reaction to 42 ℃ for 2 min. after finish the this reaction, add 1 ㎕ of to

SuperScript^{TM} Ⅱ Reverse Transcriptase and mix by pipetting gently. Incubate at 42 ℃ for 50 min and for the inactivation of to SuperScript^{TM} Ⅱ Reverse Transcriptase, heating at 70 ℃ for 15 min.

Design of primer using CODEHOP system

Collect the several species ghrelin amino acid sequence to design of primer. *Danio rerio* (Zebra fish), *Dicentrarchus labrax* (European seabass), *Micropterus salmoides* (Largemouth bass), *Oncorhynchus mykiss*(Rainbow trout), *Oreochromis mossambicus* (Mozambiqe tilapia) are selected to design of primer. And alignment to this species (figure 2). Use to that result, CODEHOP primer are designed to website (http;//blocks.fhcrc.org/blocks/codehop.html)

Table 2. Multiple alignment of another species.

Species	Position	Sequence	Score
Danio rerio	(5)	CRASSMFLLLCVSLSLCLESVSGGTSFLSPTQKPQGR	100
Dicentrarchus labrax	(4)	KKNTCLLVVLLCSLTLWCKSTSAGSSFLSPSQKPQSR	57
Micropterus salmoides	(4)	KRNTCLLVFLFCSLTLWCKSTMAGSSFLSPSQKPQSR	57
Oncorhynchus mykiss	(4)	KRNTGLMILMLCTLALWAKSCSAGSSFLSPSQKPQVR	70
Oreochromis mossambicus	(4)	KRNTCLLAFLLCSLTLWCKSTSAGSSFLSPSQKPQNK	58
Danio rerio	(61)	EDDRFMMSAPFELSMSLSEAEVEKVGPVLQNLLEDLLRD	100
Dicentrarchus labrax	(59)	ENNHITISAOFEIGVTVREEDFEECGCALQEIIQHLLGN	63
Micropterus salmoides	(59)	EDNHITISAPFEIGITMSGEDFEEVGVLLQEIIQRKKGN	57
Oncorhynchus mykiss	(68)	EDKHNTIKAPFEMGITMSEEEFQEVGAVLQKILQDVLGD	73
Oreochromis mossambicus	(59)	EDKTITLSAPFEIGCTLRAEDLADYIVELQEIVQTLLGN	74

PCR cloning of partial cDNA using degenerated primer

Due to unknown ghrelin nucleotide sequence, degenerated primer sets were designed for PCR cloning of partial cDNA.

Before the PCR, *Accuprep*® PCR PreMix (Bioneer) was prepared. For 20 ㎕ PCR reaction volume, it was composed 1 ㎕ of cDNA from from flounder, 1 ㎕ of the ghr-CODEHOP-F primer (10 mM), 1 ㎕ of ghr-CODEHOP-F primer (10 mM), and 17 ㎕ of distilled water per *AccuPrep*® PCR PreMix tube.

The PCR amplification for ghrelin gene was performed using the following one-step cycle parameters : 35 cycles at 94 ℃ for 30 sec, 55 ℃ for 30 sec, 72 ℃ for 1min. The PCR product was analyzed on a 1.5 % agarose gel stained with EtBr, along with DNA 100 bp size marker (Bioneer).

Cloning with pGEM T-easy vector

PCR products were purified with *AccuPrep*® Gel purification Kit (bioneer) from 1.5% agarose gel and cloned into th pGEM T-easy vector (Promega). Purified PCR products were ligated into pGEM T-easy vector.

Ligation mixture (purified PCR product, 70 ng; pGEM Y-easy

vector, 40 ng; 2X ligation buffer, 5 μℓ; T4 DNA ligase, 1 μℓ; upto 10 μℓ of distilled H_2O) was incubated at 16 ℃ for 4 hrs.

The ligate was used for *E. coli* XL1-Blue transformation.

Preparation of competent cell

The *E. coli* strain XL1-Blue [F'::Tn10 *pro*A^+B^+ *lac*I^q Δ(lacZ) M15/*recA1 endA1 gyr*A96 (Nal^r) *thi hsd*R17 (r_k^- m_k^+) *supE44 relA1 lac*] was incubated into a flask containing LB medium. The cell was incubated at 37 ℃ with moderated agitation until the cell density was OD_{600} = 0.5. the cultured cell was collected in to 50 mℓ centrifuge tubes such as Cornical tubes and was chilled on ice 10 min. The cell was pelleted by centrifugation at 1,000 X g for 15 min at 4 ℃.

The pelleted cells were drained thoroughly by inverting the tubes on paper towels and rapping to remove any liquid. The cell pellet was resuspended by moderate vortex mixing with 1/3 volume of TB buffer. The cells were incubated on ice for 15 min and were pellet again with same methoddescribed above. The cells were resuspended with 1/10 volume of TB buffer and were incubated on ice for 15 min.

The cells aliquoted into chilled 1.5 mℓ microcentrifuge tubes and flash freezed in liquid nitrogen and then placed at -70 ℃ Deep Freezer.

Transformation

The tubes containing competent cell were removed from the Deep Freezer and were thawed at room temperature until the cell suspension was just liquefied and were placed on ice.

The DNA solution was added to the tubes and the tube was swirled to mix the DNA with the cells. After the tubes were incubated on ice for 10 min, the cells were hear shocked by placing the tubes at 42 ℃ water bath for 55 sec and then chilled by returning the tubes immediately to ice. 900 ㎕ of LB medium was added and was incubated at 37 ℃ for 45 min. The cells were plated in LB agar plate containing ampicillin (60 ㎍ / ㎖) and the plate was incubated at 37 ℃ for overnight.

Plasmid DNA extraction

Cloned plasmid DNA was purified with *AccuPrep*® plasmid Extraction Kit (Bioneer). Cultured cell 1.5 ㎖ in LB broth containing ampicillin (60 ㎍ / ㎖) was centrifuged at 13,000 rpm for 1 min. Pellet was resuspended with 250 ㎕ of Resuspension buffer (RS) and Mixed by vortexing.

The cell was dissolved in 250 ㎕ of lysis buffer and mixed by gently inverting several times, and the lysate was incubated at room

temperature for 5 min.

After neutralized with 350 μℓ of Neutralization buffer, the lysate was mixed by gently inverting several times and centrifuged at 14,000 rpm, 4 ℃ for 10 min.

The supernatant was transferred to the binding column tube and centrifuged at 13,000 rpm for 1 min. The filtrate was poured out from collection tube and the column was washed with 700 μℓ of washing solution by centrifugation for 1 min and dried by additional centrifugation at 13,000 rpm for 1 min to completely remove ethanol. The binding column tube was transferred to sterilized 1.5 mℓ microcentrifuge tube.

The plasmid DNA was eluted by adding 100 μℓ of Elution buffer to the binding column tube and centrifuged at 13,000 rpm for 1 min.

Purified DNA was digested with EcoR I restriction enzyme (0.1 μg of cloned plasmid DNA, 1 μℓ of 10 X restriction M buffer, 1 μℓ of EcoR I m distilled water upto 10 μℓ) and analyzed on a 1.5 % agarose gel staining with EtBr, along with 100 bp size marker (bioneer).

Sequence analysis

The clone was amplified with T7 and SP6 promoter primer located at pGEM T-easy vector and seqyenced by ABI 3700 DNA analyzer from Genotech (Korea). The resulting sequences were aligned with

GenBank darabase through the BLAST search (http://www.ncbi.nlm.nih.gov/BLAST/)

First-strand cDNA

First-strand cDNA was constituted BD PowerScript Reverse Transcriptase (clonetech) following the manufacturer's protocol.

In 5'-RACE PCR of ghrelin gene 3 ㎕ of poly(A) RNA, each 12 ng of 5' CDS primer and BD SMART Ⅱ A oligonucleotide were added and sterile H_2O to final volume 5 ㎕ to a nuclease-free microcentrifuge tube. In 3'-RACE PCR of ghrelin gene 3 ㎕ of poly(A) RNA, 12 ng of 3'-CDS primer was added and sterile H_2O to a final volume 5 ㎕ to a nuclease-free microcentrifuge tube. The each mixed contents were incubated at 70 ℃ for 2 min and quickly chilled on ice. After briefly spin down, 1 ㎕ dNTP mix (10 mM), 2 ㎕ of 5 X First-Strand Buffer, 1 ㎕ DTT (20 mM) and BD PoweScript Recerse transcriptase were added and mixed by gently pipetting. The each mixed contents were spined and incubated at 42℃ for 1.5 hr in a hot-lid thermal cycler. After the reaction was inactivated by heating at 72 ℃ for 7 min, the cDNAs was used as template for RACE reaction.

Rapid amplification of cDNA ends (RACE)

Before the primary PCR, PCR Master Mix prepared. For each 50 μℓ PCR reaction, it was composed 41.5 μℓ of 34.5 μℓ of PCR-Grade Water (Clontech), 5 μℓ of 10 X BD Advantage 2 PCR Buffer, 1 μℓ of dNTP (10 mM) and 1 μℓ of 50 X BD Advantage 2 Polymerase Mix per reaction tube. 2.5 μℓ of each RACE-Ready cDNA, 5 μℓ of UPM (Universal primer Mix; 10 mM), 1 μℓ of RACE GSP1 primer (10 mM), and 41.5 μℓ of the primary PCR master mix were added to each tube.

The primary PCR was performed using the following three-step cycle parameters; 5 cycles at 94 ℃ for 30 sec, 72 ℃ for 3 min, 5 cycles at 94 ℃ for 30 sec, 70 ℃ for 3 min, and 20 cycles at 94 ℃ for 30sec, 68 ℃ for 30 sec, 72 ℃ for 3 min. the primary PCR product was analyzed on a 1.5 % agarose gel stained with EtBr, along with DNA 100 bp size marker (Bioneer).

Before the mested PCR, PCR Master Mix prepared and 5 μℓ of the primary PCR products were diluted into 245 μℓ of Tricine-EDTA buffer (Clontech). For each 50 μℓ PCR reaction, it was composed 43 μℓ of 36 μℓ of PCR-Grade Water (Clontech), 5 μℓ of 10 X BD Advantage 2 PCR Buffer, 1 μℓ of dNTP (10 mM) and 1 μℓ of 50 X BD Advantage 2 Polymerase Mix per reaction tube. 5 μℓ of each diluted primary PCR product, 1 μℓ of NUP (Nested Universal primer; 10mM), 1 μℓ of the nested PCR was performed using the following

one-step cycle parameters; 20 cycles at 94 ℃ for 30 sec, 68 ℃ for 30 sec, 72 ℃ for 3 min. The nested PCR product was analyzed on a 1.5 % agarose gel staind with EtBr, along with DNA 100 bp size marker (Bioneer).

Gradually cloning with pGEM® T-easy vector, transformation, plasmid DNA extraction and sequence analysis was processed to manual above.

Isolation of genomic DNA

The olive flounder (*Paralichthys olivaceus*) was purchased from Hae-Byun fish market. The genomic DNA of the olive flounder was prepared by AccuPrep® Genomic DNA Extraction Lit (Bioneer). 100 mg of the fresh liver was placed in liquid nitrogen and grinded to a fine powder with mortar and pestle under liquid nitrogen, placed them within 200 μℓ of Tissue Lysis buffer in a new microcentrifuge tube. After that RNase (final concentration : 20 μg/ mℓ) was added and incubated at 37 ℃ for 1 hr. Proteinase K (final concentration : 10 μg / mℓ) was added to the sample tube, mixed by vortex mixer and incubated at 60 ℃ for 2 hr. The tube was briefly spined down and added 200 μℓ of Binding buffer, and immediately mixed by vortex mixer. The tube was incubated at 60 ℃ for 10 min. 100 μℓ of isopropanol was added and the sample was mixed by pipetting and the lysate was carefully transferred into the upper reservoir of

the binding column tube and centrifuged at 8,000 rpm for 1 min. The Binding column tube was transferred to a new 2 mℓ tube for filtration. 500 μℓ of Washing buffer 1 was added to the Binding column tube without wetting the rim, and centrifuged at 8,000 rpm for 1 min. The solution was poured into a disposal bottle and 500 μℓ of Washing buffer 2 was added to the Binding column tube without wetting the rim, and centrifuged at 8,000 rpm for 1 min. The Binding column tube was centrifuged once more at 12,000 rpm for 1 min to completely remove ethanol, and transferred to a new 1.5 mℓ tube for elution, and 200 μℓ of elution buffer was added onto Binding column tube.

The sample tube was waited for 1 min at room temperature, and centrifuged at 8,000 rpm for 1 min to elute DNA. The isolated genomic DNA was stored at 4 ℃ until use.

PCR cloning of full length ghrelin genomic DNA using ghrelin specific primer.

Due to unknown ghrelin intron region nucleotide sequence, ghrelin specific primer set were designed based on RACE product.

Before the PCR, *AccuPrep*® PCR PreMix (Bionner) was prepared. For 20 μℓ PCR reaction volume, it was composed 1 μℓ of genomic DNA (30 μg /mℓ) from flounder, 1 μℓ of ghr-specific-F (10mM), 1 μℓ of ghr-5'RACE-GSP 1 primer (10 mM), and 17 μℓ of distilled

water per *AccuPrep*® PCR PreMix tube.

The PCR amplification for ghrelin gene was performed using the following one-step cycle parameters : 40 cycles at 94 ℃ for 40 sec, 55 ℃ for 40 sec, 72 ℃ for 4min. The PCR product was analyzed on a 1.5% agarose gel stained with EtBr, along with DNA 100 bp size marker (Bioneer)

Primer walking of genomic DNA

Genomic DNA of flounder were used as template. Primer walking was conducted using DNA walking DNA Walking *SpeedUp*™ Premix Kit (Seegene) following the manufacturer's protocol. Before the first PCR, PCR master mix prepared. For each 50 ㎕ PCR reaction, it was composed 43 ㎕ of 18 ㎕ of distilled water and 25 ㎕ of 2 X SeeAMP™ ACP™ Master Mix Ⅱ per reaction tube. 2 ㎕ of genomic DNA, 4 ㎕ of 2.5 pmol DW-ACP (one of DW-ACP 1, 2, 3, and 4) primers and 1 ㎕ of primer walking GSP 1 primer (10 mM) were added to each tube. The first PCR was performed using the following two-step cycle parameters : 1 cycle at 94 ℃ for 5 min, 42 ℃ for 1 min, 72 ℃ for 2 min and 30 cycles at 94 ℃ for 40 sec, 50 ℃ for 40 sec, 72 ℃ for 2 min. The first PCR products were purified using *AccuPrep*® PCR Purification Kit (Bioneer).

Before the second PCR, PCR master mix prepared. For each 20 ㎕ PCR reaction, it was composed 15 ㎕ of 5 ㎕ of distilled water and

10 μℓ of 2 X SeeAmp™ ACP™ Master Mix Ⅱ per reaction tube. 3 μℓ of purified first PCR products, 4 μℓ of DW-ACPN primer (10 mM) and 1 μℓ of primer walking GSP 2 primer (10 mM) were added to each tube. The second PCR was performed using the following one-step cycle parameters : 35 cycles at 94 ℃ for 40 sec, 55 ℃ for 40 sec, 72 ℃ for 2 min. The second PCR products were purified using *AccuPrep*® PCR Purification Kit (Bioneer) and analyzed on a 1.5% agarose gel stained with EtBr, along with DNA 100bp size marker (Bioneer).

Gradually cloning with pGEM® T-easy vector, transformation, plasmid DNA extraction and sequence analysis was processed to manual above.

Primer extension for identification of transcription start site

For identification of Ghrelin gene' transcription start site, process the primer extension method. Ghrelin specific primer design based on ghrelin sequence, and primer were modified to 5' phosphate (ghr-ext-R1 and ghr-ext-R2).

For the primer labeling, preparation of microcentrifuge tube, add to 2 μℓ of specific primer (10pmol) or Control primer, 1 μℓ of T4 PNK 10 X buffer, 3 μℓ of [γ-^{32}P]ATP (3,000 Ci/ mmol, 10 mCi/ mℓ), 2 μℓ of T4 polynucleotide Kinase (10 u/ μℓ), and 2 μℓ of nuclease-free water. And then, incubate at 37 ℃ for 30 min. heat to 90 ℃ for 2

min to inactivate the T4 PNK, then centrifuge briefly in a microcentrifuge. Bring the final concentration of the Control primer to 100fmol/ µℓ by adding 90 µℓ of nuclease free water.

For the DNA Marker labeling, add to 5 µℓ of *ϕX174DNA/Hinf I* dephosphorylated makers (250 ng), 1 µℓ of T4 PNK 10 X buffer, 3 µℓ of [ɣ-^{32}P]ATP(3,000 Ci/ mmol, 10 mCi/ mℓ), and 2 µℓ of T4 polynucleotide Kinase (10 u/ µℓ) in microcentrifuge tube. Incubate at 37 ℃ for 30 min. Heat to 90 ℃ for 2 min to inactivate the T4 PNK. Centrifuge briefly in a microcentrifuge and add 190 µℓ of nuclease free water and store at -20 ℃. Loading 1 µℓ of the diluted phosphorylated markers onto a gel will provide dark bands on an autodiogram following overnight exposure of the gel film.

Primer extension of control and sample RNA are start to following state. Combine the components for primer annealing in microcentrifuge tube. Added 1 µℓ of ^{32}P labeled specific primer and 5 µℓ of AMV primer extension 2 X buffer. Mix by gently pipetting and dilute the Control RNA in Nuclease-Free Water to provide desired amount of RNA per 5 µℓ aliquot. The sample RNA may or may not need to be diluted to obtain the desired amount of RNA per 5 µℓ aliquot.

Annealing the primer and RNA by heating the tubes at 58 ℃ (control primer) for 20 min to 1 hr followed by cooling at room temperature for 10 min. the 58 ℃ temperature is optimal for annealing the control primer and control RNA. The optimal

temperature for other primers and templates may be different. In general, the optimal annealing temperature should be at or near the melting temperature (Tm) for the primer. Time may be extended from 1 hour to overnight.

Pre-warm the 40 mM Sodium Pyrophosphate, Nuclease-Free Water and AMV primer Extension 2 X Buffer to room temperature or 37 ℃. Combine the components for a “master” reverse transcriptase (RTase) extension mix. Add the AMV RT last and mix by pipetting and by gently inverting the tube. Mixture are contained to 5 μℓ of AMV primer Extension 2 X buffer, 1.4 μℓ of Sodium Pyrophosphate(40mM), 1 u of AMV RT and 9 μℓ of Nuclease Free Water in per reaction.

Incubate at 42 ℃ for 30 min, and ended to this reaction, added 20 μℓ loading dye to each tube. Heat the tubes (including the markers) at 90 ℃ for 10 min and loading samples directly onto a gel. A typical volume loaded is 20 μℓ of the 40 μℓ volume. Store the remainder of the samples at -20 ℃.

Gel for electrophoresis was composed the 7 M urea, 8 % polyacrylamide gel. Electrophoresis was performed 160 V at 40 min.

RESULTS AND DISCUSSION

Nucleotide sequence of ghrelin gene in olive flounder

Ghrelin gene using a flounder stomach cDNA was cloned as a template by polymerase chain reaction (PCR).

As shown in Figure 1. (A), PCR products about 145 bp (ghrelin gene) a respectively obtained in cDNA of olive flounder owing to underexposed sequence database. Ghrelin fragment was obtained using ghr-CODEHOP-F and ghr-CODEHOP-R primers. These primers were designed through alignments of ghrelin gene of *Danio rerio* (Zebra fish), *Dicentrarchus labrax* (European seabass), *Micropterus salmoides* (Largemouth bass), *Oncorhynchus mykiss*(Rainbow trout) and *Oreochromis mossambicus* (Mozambiqe tilapia). The nucleotide sequence of partial ghrlein gene were sequenced as shown in Figure 1. (B), and analysis using the Genbank database.

Partial fragment size of ghrelin gene was short to RACE analysis, consequently as shown in Figure 2. (A) two 3' RACE products, 477 bp and 756 bp band are obtained using 3' RACE kit (BD PowerScript Reverse Transcriptase kit, clonetech) from olive flounder stomach cDNA. Also, Figure 2. (B) 5' RACE product was shown size of 507 bp band. Revealed three bands sequence and join the overlapping region, we have to ghrelin full length ORF (Open Reading

Frame).(Figure 3.)

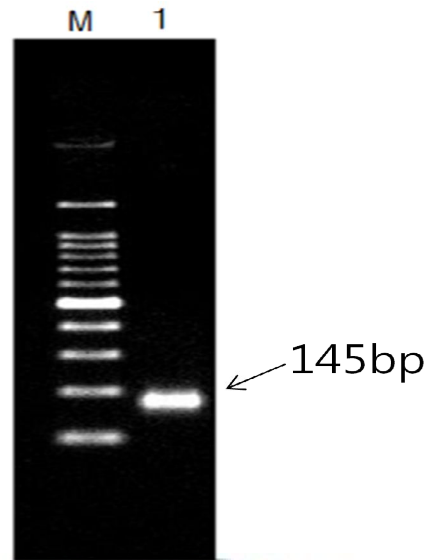
As shown in Figure 4, size of 1,544 bp band were optained using primer walking system (DNA Walking *SpeedUp*™ premix Kit, Seegene) from olive flounder genomic DNA.

Nucleotide and deduced amino acid sequence was optained as shown in Figure 5 by sequencing service (Genotech).

Compared ghrelin ORF resion and full length ghrelin genomic DNA sequence, revealed the ghrelin has 3 introns and 4 exons (Figure 6.).

Transcription start site was revealed to compare 5' RACE result and primer extension result. Two result was almost consensus.

A.



1.5 % agarose gel
stained with EtBr

B. Partial nucleotide sequence of ghrelin.

1	CCAGCTTTCTCAGCCCGTCACAGAAACCGCCGAACAAGGGGAAACCTCCCAGACCCGGCC	60
61	GCCAAATCACAGAGGAGCAGAGTCAACACACCGAGGACCACCACATCACAGTAAGTGCAC	120
121	CGTTTGAAATGTCCGTCTGCGAAAA	145

Figure 1. The result of PCR from partial cDNA using CODEHOP primer. (A) M; 100 bp DNA ladder (Bioneer), 1; PCR products amplified with ghr-CODEHOP-F and ghr-CODEHOP-R in cDNA. (B) partial nucleotide sequence of ghrelin gene in olive flounder cDNA.

A. B.

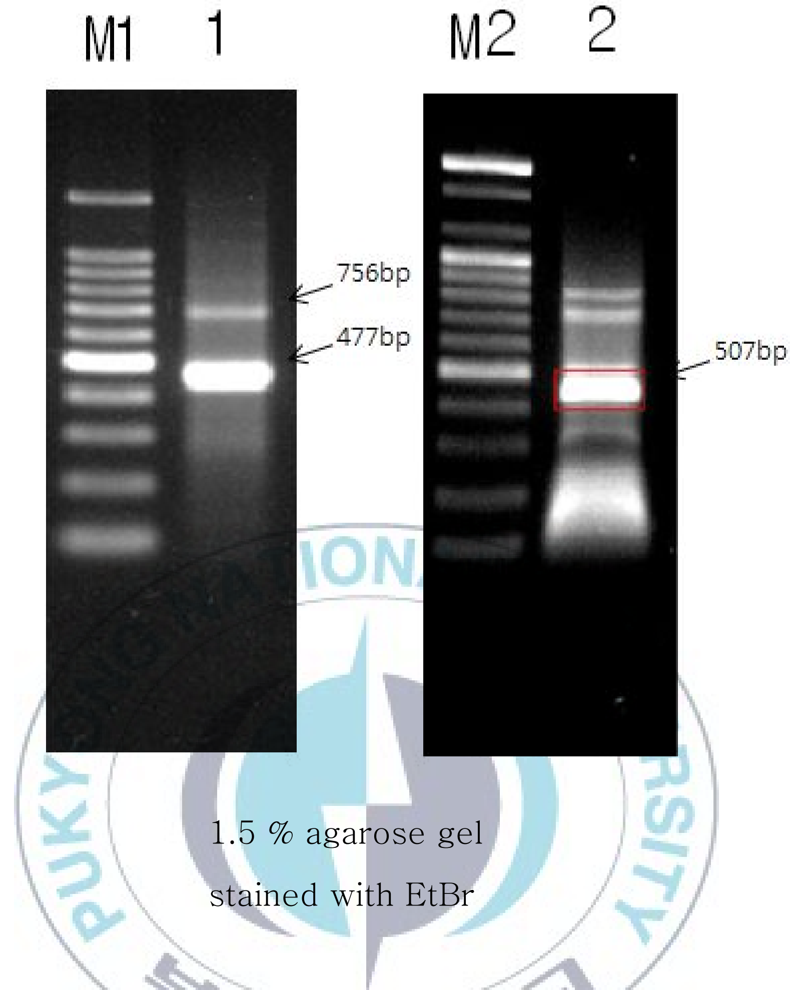
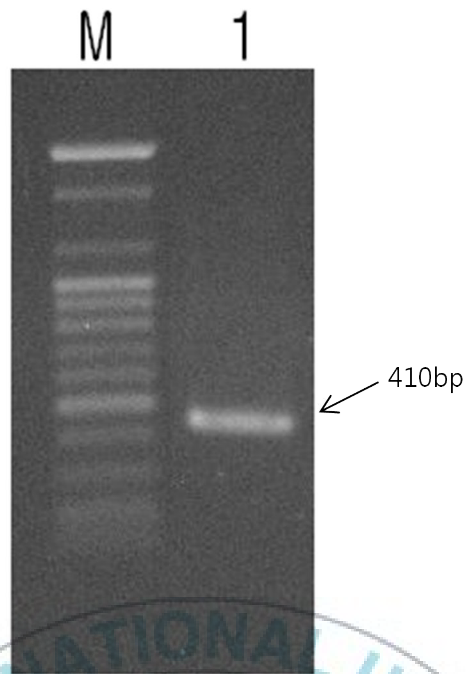


Figure 2. The results of 3' RACE and 5' RACE. (A) M1; 100bp-1.5 Kb DNA ladder (Biosesang), 1: 3' RACE products (477 bp and 756 bp) of ghrelin gene using NUP and ghr-3' RACE GSP 2. (B) M2; 100bp DNA ladder (Bioneer), 2; 5' RACE product (507 bp) of ghrelin gene using NUP and ghr-5' RACE GSP 3.



1.5 % agarose gel
stained with EtBr

Figure 3. The result of PCR from ORF of ghrelin gene. M; 100 bp DNA ladder (Bioneer). 1; PCR product amplified with ghr-specific F and ghr-5' RACE-GSP 3.

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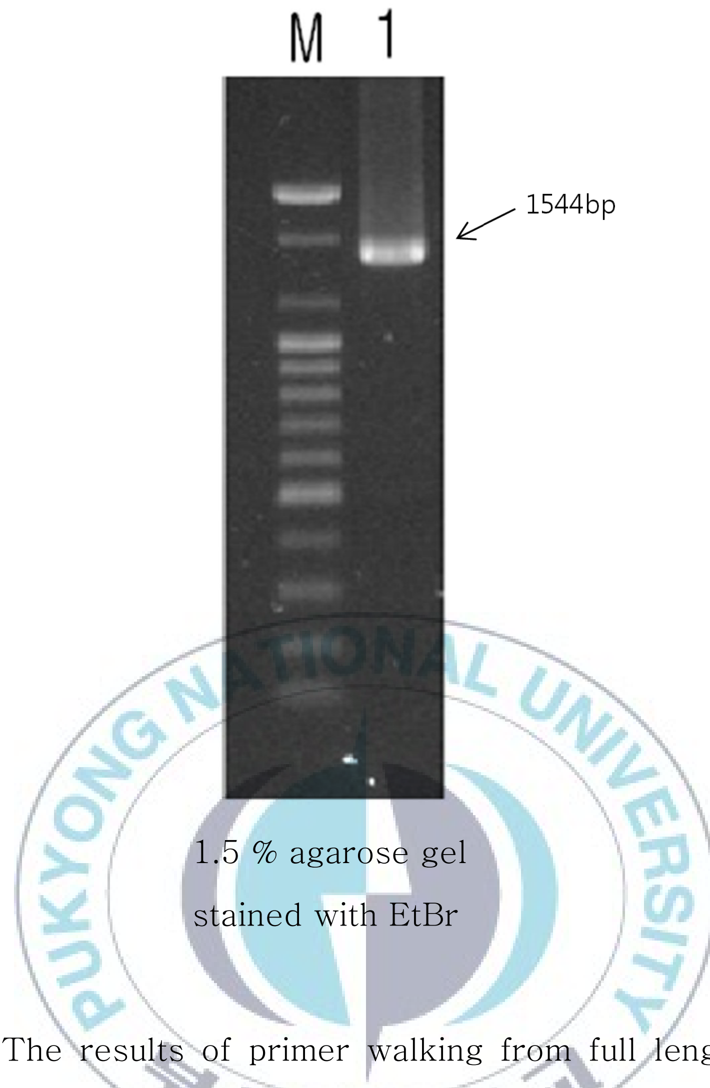

1.5 % agarose gel
stained with EtBr

Figure 4. The results of primer walking from full length genomic DNA. M; 100bp DNA ladder (Bioneer) 1; PCR products amplified with ghr-specific F and ghr-5' RACE-GSP.

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```
     ttaacgctatacgttgcttcgtcatttcgcagcggagacttcggttccacc
       M  F  L  K  R  N  T  R  L  L  V  F  L  L  G  S  L  T  L  W     20
  1  ATGTTTCTGAAAAGAAACACCAGGCTGCTGGTTTTTCTACTCGGTTCTC GACCTTGTGG
       C  K  S  T  S  A  G  S  S  F  L  S  P  S  H  K  P  P  N  K     40
 61  TGTAAGTCGACCAGCGCAGGCTCCAGTTTTCTCAGCCCTTCACACAAACCGCCGAACAAG
       G  K  P  P  R  P  G  R  Q  I  T  E  E  Q  S  Q  H  T  E  D     60
121  GGGAAACCTCCCAGACCCGGCCGCCAAATCACAGAGGAGCAGAGTCAACACACCGAGGAC
       H  H  I  T  V  S  A  P  F  E  I  G  I  T  M  T  P  E  D  F     80
181  CACCACATCACAGTAAGTGCCCCATTTGAAATTGGCATCACCATGACACCAGAGGACTTT
       E  E  Y  G  M  L  L  Q  E  I  V  Q  R  L  L  G  N  T  E  T    100
241  GAGGAGTATGGCATGTTGCTGCAAGAGATCGTTCAGCGTCTGCTGGGAAACACGGAGACG
       A  E  R  P  S  *                                                105
301  GCAGAGAGGCCATCCTAA
     ctttgaatattatggacacgactggcagatttactgctttatttctttcaatttctactt
     agatagtggtcattaaaatatgagtcaacgtgtcagtaaccttaagcttaaagatttaaa
     acactattactcactgttggtagttcatctgtacaattaacactagagacatattcatca
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     gatttaagtctcacataagcttccattattaggttattttattttcaaaactaacccagt
     gctagcagaataattactcattcatacctatatgatgaactacacccccctctgtagtaca
     gaatataacatacaataagtccttgtcttcagagactttacacgtggtggtcaaaataaa
     gcatcagt
```

Figure 5. The nucleotide and deduced amino acids sequence of cDNA encoding ghrelin gene in olive flounder.

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ttaacgctat acgttgcttc gtcatttcgc agcggagact tcggttccac c

5' UTR ATGTTTCTG 60

AAAAGAAACA CCAGGCTGCT GGTTTTTCTA CTCGGTTCTC TGACCTTGTG GTGTAAGTCG 120

ACCAGCGCAG GCTCCAGTTT TCTCAGCCCT TCACACAAAC CGCCG

Exon 1 gtaag atgctttctg 180

ggattcatta tgaaacaaca gttaaaaata gaggcgtgaa tcctcccatg agcttaactt 240

tcaacataac tcaatatacc ataataaagg acttattcat ttgttttaaa ctgtctgatg 300

tttcaaaaat ctatatttta cttgttcagc cttacaacgt accacactta actaaactcc 360

tgccactgaa tccaatcttt gatttccagc aagtttcaaa atgtaatctg ttgagtacat 420

intron 1

ttttgctgag aatggtgtta tgactaaaat ggattttgtc agtatgcagg tataattttc 480

tataatttca aatgcatgca ttgtgttctc cagtgtattc aagacatgag aaaaccttgc 540

aattgaacta ctatcatgaa actgaattgg ttacaatcat gagatatgta tgttggtcaa 600

acacag

AACA AGGGGAAACC TCCCAGACCC GGCCGCCAAA TCACAGAGGA GCAGAGTCAA 660

CACACCGAGG ACCACCACAT CACAGTGAGT G

Exon 2 gaaaatata acacacttgt gacaacagca 720

aagcatcaaa catcatccct gtgtctttat ggtcttaagc aggtaagtg

Intron 2 C CCCATTTGAA 780

ATTGGCATCA CCATGACACC AGAGGACTTT GAGGAGTATG GCGTGTTGCT GCAAGAGATC 840

GTTCAGCGTC TGCTGGGAAA CACGGAGACG GCAG

Exon 3 gtacac acacaacccc ctctgtagtt 900

gtgtttcctg gaccaatgca tggtaagata atgatctgac cattttaat gttattttaa 960

intron 3

actattgtct cttttttttc ag

AGAGGCCA TCCTAA

Exon 4

cttt gaatattatg gacacgactg 1020

gcagatttac tgctttattt ctttcaattt ctacttagat agtggtcatt aaaatatgag 1080

tcaacgtgtc agtaacctta agcttaaaga tttaaaacac tattactcac tgttggtagt 1140

tcatctgtac aattaacact agagacatat tcatcaagtg aaactatgag tatgtttatt 1200

ttcagagctt tcaaccacat ctcaatgtca ctatcagtta gaaatggttg tgttaaaaat	1260
aataaagaag ttaatatcaa ttcaatatct ataatttgaa acttgcgttt gaattatttt	1320
3’ UTR	
gtcaaactat ggttctcatt catgaattta gcgctgagat ttaagtctca cataagcttc	1380
cattattagg ttattttatt ttcaaaacta acccagtgct agcagaataa ttactcattc	1440
atacctatat gatgaactac accccctctg tagtacagaa tataacatac aataagtcct	1500
tgtcttcaga gacttacacg tggtggtcaa aataaagcat cagt	1544

Figure 6. Location of exon and intron of ghrelin gene in genomic DNA

A.

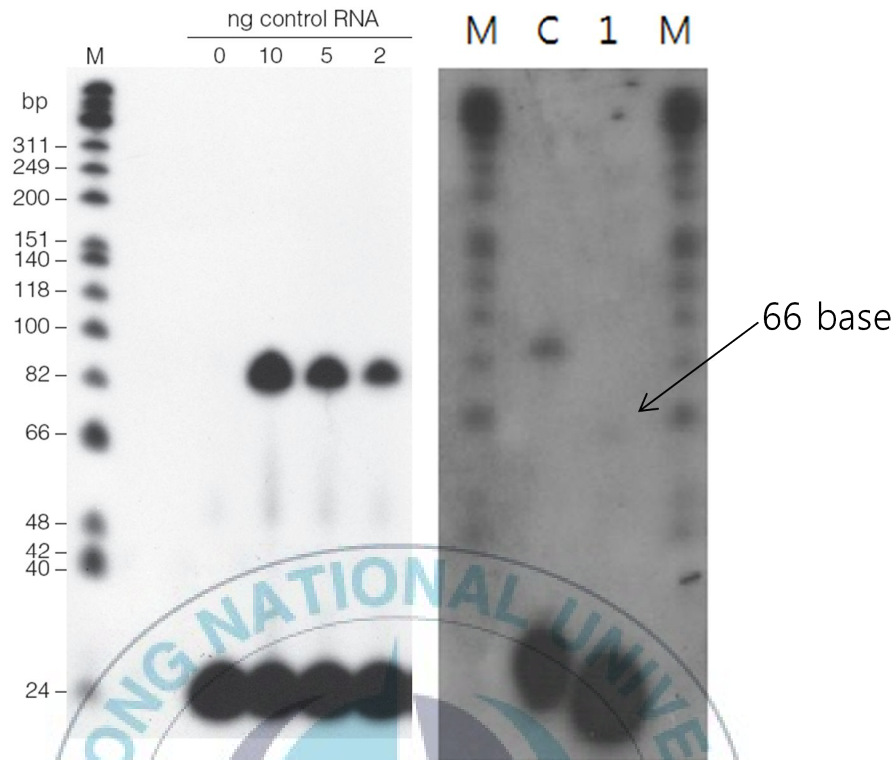

Figure 7. Result of primer extension and identification of transcription start site compare 5' RACE result and primer extension result. (A) M; *ϕX174DNA/ Hinf I* dephosphorylated maker. C; control RNA, 1; result of primer extension using ghr-ext-R2 primer. (B) Identification of transcription start site to compare 5' RACE and primer extension. This sequence was revealed the genome walking method. a was shown the primer extension result (-66 base), and b was shown the 5' RACE result.

B.

```
-379                      GGAG TTTAGGTCCA GCGTCCGTGG GGGCAGTCCA TTAAGTAATA
-335  GACAAGTCCT GTAATATTCT CGTACACATG CAGAATAGAC TAGATCTCTG TGACTGCAAT
-275  GCTATTACCC TCATTACCCA ACTGAATAGA TATGATGATC AGCAGGTCAA CAGAGAAATG
-215  TCCACTTTGT TATCAATTCA AACATTTGAA CAGTCCAATG CTTCCTAGAT ATATCTGTTT
-155  AATGCATTTT AAATCTGCAT GAAAGGGGAT GGAGGTCAGG CTATATAAAC AGAACTACAA
 -95  GTGTCAGTGT CAGATTTAGA GCAGCGGATT TGATTTGAAA TCTTTTAACG CTATACGTTG
                                  a.                b.
 -35  CTTCGTCATT TCGCAGCGGA GACTTTGGTT CCACCATGTT TCTGAAAAGA AACACCAGGC
```

Figure 7. continue

Amino acid sequence analysis and phylogenetic tree.

Deduced amino acid sequence of ghrelin was respectively analyzed for multiple alignment, and draw phyrogenetic tree (Cluster W 2.0).

The multiple alignment, phylogenetic tree were shown in Figure 8,and 9 using a deduced amino acid sequence of cDNA encoding ghrelin.

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Figure 8. Multiple alignment of the deduced amino acid sequenc of ghrelin with known ghrelin protein sequence taken from GenBank.

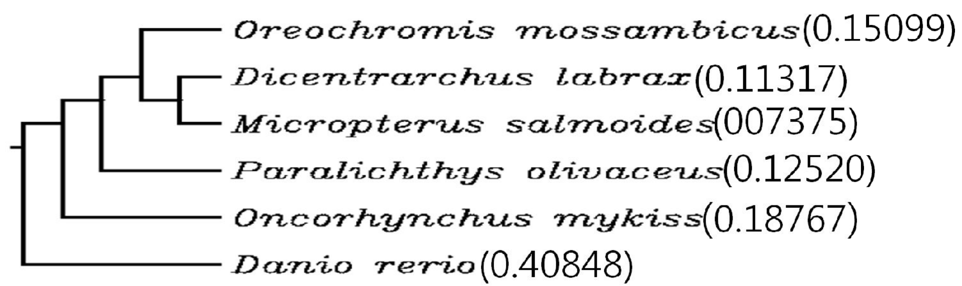

Figure 9. A molecular phyrogenetic tree of ghrelin amino acid based on the Neighbor joining method. Numbers in parentheses are brench lengths.

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국문 초록

양식 넙치의 ghrelin 유전자의 특성

정 영 식

부경대학교 대학원 미생물학과

대표적인 양식어종인 넙치 (*Paralichthys olivaceus*)의 ghrlein 유전자는 105개의 아미노산으로 구성된 호르몬으로서, 이 호르몬은 성장호르몬을 분비를 증가시키는 기능을 가지고 있는 것으로 확인되었다. 또한 ghrelin은 인슐린의 분비를 조절하여 혈액 속의 당 농도를 조절하는 기능이 있다고 확인되었다. 본 논문에서는 넙치의 ghrelin 유전자의 특성을 연구하였다. Ghrlein 유전자를 cloning 하기 위하여 zebrafish, European seabass, Mozambique tilapia등의 ghrelin 유전자의 부분 염기서열에서 특이적인 프라이머 조합을 합성하고 넙치의 위로부터 rapid amplification of cDNA ends (RACE)를 실시하여 308bp, 105개 아미노산을 암호화하는 open reading frame (ORF)를 확보하였다. 또한 앞의 rapid amplification of cDNA ends (RACE)의 염기서열에서 특이적인 프라이머 조합을 합성하였고, 넙치의 genomic DNA에서 genome waling 방법을 사용하여 1544bp의 full length ghrelin genomic DNA을 확인하였다. Full length ghrelin genomic DNA에서 Exon은 4개가 존재하였으며, Intron은 3개가 존재하는 것을 확인하였다.

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부족한 저에게 세심하게 신경 써 주시며, 저에게 마지막까지 옆에서 많은 가르침을 주신 정인영 선배님께 감사드립니다. 실험과 대학생활의 생활에 있어서 많은 조언과 도움을 주신 서용배, 정혜은, 지근호, 이경용, 최성석, 정대혁, 장지원, 윤진수, 임지영, 배성자, 황희주, 김상보, 정현일, 김현수, 박미선, 김아름 각 실험실 선배님과 동기들, 후배님들께 정말 감사드립니다.